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Prospective Multicenter Study of *Pneumocystis jirovecii* Colonization among Cystic Fibrosis Patients in France

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***Pneumocystis* carriage was detected in 12.5% of 104 cystic fibrosis (CF) patients during a prospective multicenter French study, with a prevalence of genotype 85C/248C and geographic variations. It was significantly associated with the absence of *Pseudomonas aeruginosa* colonization and a greater forced expiratory volume in 1 s. Results are discussed considering the natural history of CF.**

While *Pneumocystis jirovecii* is a well-known cause of pneumonia (PcP) in severely immunocompromised patients, *Pneumocystis* carriage has drawn increasing interest. Several works support a significant association of *P. jirovecii* colonization with diverse chronic pulmonary diseases (6, 12, 21). Few severe PcP cases in cystic fibrosis (CF) patients have been reported (23, 25), and only some studies have reported *Pneumocystis* carriage, the clinical significance of which remains practically unexplored (14, 18–20, 22–26). The aims of the present study were therefore to evaluate the prevalence of *P. jirovecii* colonization during a prospective multicenter observational study, to provide information regarding the distribution of *Pneumocystis* genotypes, and to explore the clinical relevance of *Pneumocystis* colonization among CF patients in France.

One hundred four CF patients with a median age of 24.0 years (interquartile range: 18 years in quartile 1 [Q1] to 29.5 years in Q3; 50 males and 54 females) were included by physicians according to the same criterion (an annual checkup or an exacerbation situation that required an expectorated sputum sample) and were screened for *P. jirovecii* carriage (reference number of the institutional ethics committees of Lille Hospital, CPP 06/84). All patients had a well-documented diagnosis of CF with either the two known mutations in the CF transmembrane conductance regulator gene (35.6% were homozygous and 45.2% were heterozygous for the F508del mutation) or an abnormally high sweat chloride test result (median, 102 mmol/liter; Q1-to-Q3 range, 90 to 128 mmol/liter). Clinical data, including spirometric, therapeutic, radiological, and biological data, were collected by clinic staff at each visit and used for statistical analysis with SAS software (version 9.2; SAS Institute). Sputum specimens ($n = 146$) were collected from hospitals in Lille (93 samples, 58 patients), Dunkerque (27 samples, 20 patients), Angers (18 samples, 18 patients), and Bordeaux (8 samples, 8 patients) between October 2006 and March 2009. Each specimen examined was considered an independent event since the delay between two sputum sample collections was at least 6 months (18). Samples were collected and analyzed according to a standardized protocol as previously described (3, 8).

After DNA extraction (8), the presence of *P. jirovecii* was detected by combining an initial conventional PCR with a real-time PCR (RT-PCR). Briefly, RT-PCRs were performed with a final volume of 20 μl consisting of 18 μl of LightCycler FastStart *Taq* (Roche), reaction buffer containing 2.4 mM $MgCl_2$, a 0.5 μM concentration of each primer (pAZ 102 X and pAZ 102 Y), a 0.2 μM concentration of the fluorescein (5′-CAG ACT ATG TGC GAT AAG GTA GAT AGT CGA [Flc]-3′) and LC Red-640 (5′-[LC640] GGA AAC AGC CCA GAA CAG TAA TTA AA-3′) FRET (fluorescence resonance energy transfer) probes, and 2 μl of template DNA obtained from the first-round PCR. Initial preheating and touchdown procedures were performed on the LightCycler 2.0 system as previously described (13). Samples were handled under a laminar-flow hood. Extraction, mixture preparation, and LightCycler carousel loading were performed in different rooms. Negative and positive controls were included in each extraction step and each PCR. PCR inhibitions were identified when DNA samples were diluted 1/10. A sample was considered positive for *P. jirovecii* DNA detection when at least one combined PCR assay (with pure or 1/10-diluted DNA) yielded a positive result. Purified amplicons from positive samples were sequenced directly (18) to detect mitochondrial large-subunit (mtLSU) rRNA polymorphisms (10, 14, 16–20, 22, 24). *Pneumocystis* colonization (or asymptomatic, subclinical carriage) was defined as molecular detection (positive PCR) without positive direct examination, clinical signs of PcP, or progression to PcP infection (4, 5).

P. jirovecii DNA was detected in 13/104 (12.5%) patients, corresponding to a total of 17 positive samples. In five samples, PCR inhibitions were observed and avoided the use of 1/10-diluted DNA. Patients colonized with *Pneumocystis* were distributed as follows: four patients from Angers, two from Bordeaux, four from Dunkerque, and three from Lille. The colonization rate reported

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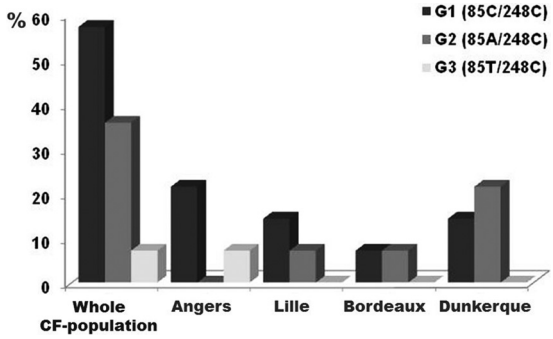

FIG 1 Distribution of *P. jirovecii* mtLSU rRNA genotypes (G1 to G3) in French CF patients according to the geographic origins of the patients.

in Lille (3 patients out of 58 followed up at Lille Hospital, 5.2%) was significantly lower than that at the other centers ($P = 0.036$, Fisher's exact test). Fourteen out of the 17 PCR-positive samples were successfully sequenced. Only mutations at position 45 were recorded, leading to a predominance of genotypes 1 and 2 (genotype 2 was sequentially isolated from two samples from one patient in Lille; Fig. 1). Their distributions varied according to the CF patients' places of diagnosis (Fig. 1).

When we compared the characteristics collected at each sampling time of CF patients with or without indication of *P. jirovecii* carriage (Table 1), *Pneumocystis* colonization was significantly associated with the absence of colonization by a *Pseudomonas aeruginosa* mucoid strain ($P = 0.020$, Fisher's exact test). Moreover, the FEV_1 (forced expiratory volume in 1 s) values (percentage of the predicted FEV_1) of patients colonized with *P. jirovecii*

TABLE 1 Comparison of characteristics of subjects with and without detectable *P. jirovecii* DNA in sputum samples at each sampling time

Characteristic	*P. jirovecii* DNA not detected in sputum samples ($n = 129$)	*P. jirovecii* DNA detected in sputum samples ($n = 17$)	*P* value[a]
General data			
No. (%) of males, females	44 (48.4), 47 (51.7)	6 (46.2), 7 (53.9)	NS
Avg age (yr), range	24, 18–32	23, 15–28	NS
CF clinical status			
Median S-K score (Q1-Q3 range)	75 (60–83)	80 (60–90)	NS
Median BMI (Q1-Q3 range)	19.1 (17.2–21.1)	19.2 (18.0–20.9)	NS
Median FEV_1 (Q1-Q3 range)[d]	53.0 (36.0–77.0)	80.0 (46.0–100)	0.032
Median FVC (Q1-Q3 range)[d]	68.5 (54.0–89.7)	90.0 (61.0–109)	NS
Microbiological data			
No. (%) colonized[e] with:			
Haemophilus influenzae	24 (18.8)	3 (17.7)	NS
Methicillin-sensitive *Staphylococcus aureus*	51 (39.8)	7 (41.2)	NS
Methicillin-resistant *Staphylococcus aureus*	24 (18.8)	0	NS
Pseudomonas aeruginosa nonmucoid strain	67 (52.3)	10 (58.8)	NS
Pseudomonas aeruginosa mucoid strain	70 (54.7)	4 (23.5)	0.020
No. (%) with mycological sputum cultures positive for:			
Aspergillus fumigatus	61 (48.0)	6 (35.3)	NS
Scedosporium apiospermum	10 (7.9)	0	NS
Candida albicans	72 (56.7)	11 (64.7)	NS
No. (%) treated with:			
Nebulized rhDNase[f]	82 (64.6)	11 (68.8)	NS
Inhaled antibiotics (continuous[b])	118 (92.2)	15 (88.2)	NS
Inhaled steroids (continuous)	64 (50.0)	9 (52.9)	NS
Azithromycin	65 (50.8)	8 (47.1)	NS
No. (%) with intermittent antibiotic exposure[c]			
Oral antibiotics (1 to 3 treatments)	98 (78.4)	14 (82.4)	NS
Intravenous antibiotics (1 to 13 treatments)	73 (59.4)	7 (41.2)	NS
Oral antifungals	33 (26.0)	3 (17.7)	NS
Systemic steroids (continuous)	28 (21.9)	5 (29.4)	NS

[a] NS, not significant ($P < 0.05$).
[b] Continuous drug exposure is defined as daily administration over the previous 6 months (3).
[c] Intermittent antibiotic exposure does not include trimethoprim-sulfamethoxazole.
[d] As a percentage of the predicted value.
[e] At least once in the preceding year.
[f] rhDNase, recombinant human DNase.

were significantly higher (80% versus 53.0% of FEV_1 median, $P = 0.032$ using the Kruskal-Wallis test), suggesting better pulmonary function in *Pneumocystis*-colonized, *P. aeruginosa*-positive patients. The present population of CF patients colonized with *Pneumocystis* was not considered to have severe airway disease, since the median (Q1-to-Q3 range) Shwachman-Kulczycki (S-K) score, body mass index (BMI), and forced vital capacity (FVC; percentage of the predicted FVC) were, respectively, 80 (60 to 90), 19.2 (18.0 to 20.9), and 90 (61 to 109%) (Table 1), the S-K score being <50%, the BMI <16, and the FVC <50% in severe disease. There was no significant association between therapeutics, including systemic corticosteroid therapy, and *Pneumocystis* colonization. Since the overall numbers of subjects in the subgroups were small, univariate methods were used here, and to confirm these results, larger studies and the use of multivariate analysis to select variable subsets and to determine the relative worth of the variables are required. The clinical and microbiological features of our population were in agreement with recently published data (2, 3).

P. jirovecii is now classified as an atypical fungus that is unable to grow *in vitro* in current fungal culture media and exhibits pulmonary tropism with strict human host specificity. While PCR assays have represented a significant advance in *Pneumocystosis* diagnosis, they also revealed *Pneumocystis* colonization occurring in immunocompromised and nonimmunocompromised patients, and allowed exploration of the corresponding *P. jirovecii* circulation between humans, including nonimmunocompromised patients (5, 6, 10, 12–15, 17–22, 24, 26–29, 32). Here, we combined conventional PCR and RT-PCR methods targeting the mtLSU gene in order to increase the detection rate while maintaining the specificity of detection by using FRET probes. It is recommended that both diluted and undiluted DNA samples be used; diluted DNA overcomes potential inhibitors, and undiluted DNA overcomes amplification failures due to scarce amounts of *Pneumocystis* DNA in the specimen. According to our observations, it is a reliable technique that allowed us to detect *Pneumocystis* colonization in 12.5% of our patients. This percentage is in agreement with data reported for CF patients from European and Brazilian areas (7.4, 21.5, and 38.2%) (22, 24, 26) but higher than the value of 1.3% reported by Le Gal et al. for a series of 76 CF patients from French Brittany (14). As previously reported (1, 17), such geographic differences in *Pneumocystis* carriage prevalence might be linked with the local *Pneumocystis* circulation, which may be lower in northern France (14; our results in Lille). The low prevalence of *Pneumocystis* carriage we observed in the Lille population could be related to the climate, which is colder than that of Angers or Bordeaux (average yearly temperatures of 10.4°C, 11.8°C, and 13.3°C, respectively), as previously suggested (16, 22). Using mtLSU rRNA (a highly informative marker for discerning associations between specific genotypes and geographical or clinical data [1, 10, 13–20, 22, 24, 26–29, 32]), we identified genotypes 1 and 2 as predominant, in agreement with data from most European and American studies of non-CF or CF patients (1, 16, 17). Taking into account all of these studies, including Spanish and Brazilian ones that have reported genotypes 1 and 3 as predominant in CF patients, genotype 1 polymorphism might be the best adapted to patients with CF (1, 16–18, 20, 22, 24).

Despite technical factors that can influence the reported prevalence of *Pneumocystis* colonization, and according to the above-cited studies (including our results), it appears that CF is associated with lower *Pneumocystis* colonization rates than are other chronic pulmonary diseases (14, 20, 22, 24, 26, 27, 32). Three studies have reported the association of *P. jirovecii* and *P. aeruginosa* in CF patients (14, 22, 25), but whether *Pneumocystis* infection/colonization complicated CF-associated lung disease remains unclear. In fact, little is known regarding the *in vivo* interactions between species, especially considering the bacterial and fungal community as a unique entity (lung microbiota) (8, 11, 31). In our study, *Pneumocystis* carriage was associated with the absence of *P. aeruginosa* and with less severe lung disease, a result in agreement with the role of *Pneumocystis* microorganisms in stimulating pulmonary inflammation, which is involved in the initial development of lung diseases and in this manner encourages other, subsequent infections, as recently proposed (6, 7, 9, 12, 21, 30). While large amounts of immunosuppressors, including steroids, seem to be a crucial factor that allows PcP to occur (4, 6, 21, 32), we did not find any association between *P. jirovecii* colonization of CF patients and systemic steroid therapy (Table 1).

To conclude, we have reported the prevalence of *Pneumocystis* carriage in CF patients from four different French areas. Whether *Pneumocystis* colonization could have a role in the natural course of CF lung disease has not been demonstrated so far, but the role of CF patients colonized by *P. jirovecii* as a reservoir and as infective sources of this microorganism for other susceptible individuals can be strongly suspected (28, 29), which is consistent with the continuous infection-and-clearance cycle involving *P. jirovecii* recently reported in CF patients by Montes-Cano et al. (18).

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